Fluorescent Labeling of RAFT-Generated Poly(*N*-isopropylacrylamide) via a Facile Maleimide−Thiol Coupling Reaction†

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We report a facile labeling technique in which the telechelic thiocarbonylthio functionality of well-defined poly(*N*-isopropylacrylamide) (PNIPAM) prepared by room temperature RAFT polymerization is first converted to the thiol and subsequently reacted with a maleimido-functional fluorescent dye, *N*-(1-pyrene)maleimide (PM). Nearly monodisperse PNIPAM (M_n = 39 500 g/mol, M_w/M_n = 1.07) was synthesized using a trithiocarbonate-based CTA, 2-dodecylsulfanylthiocarbonylsulfanyl-2-methyl propionic acid (DMP), and a conventional azo-initiator, namely, 2,2'-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70), as the primary source of radicals. The key to successful conjugation of PM to PNIPAM is the implementation of a two-step reduction process involving (1) the cleavage of the trithiocarbonate with a strong reducing agent, in this case, $NaBH_4$, to form a mixture of polymeric thiols and disulfides and (2) the conjugation of PM to the pure polymeric thiol in the presence of tris(2-carboxyethyl)phosphine·HCl (TCEP). We show that TCEP efficiently eliminates the formation of polymeric disulfides and thus allows for the desired addition of the free polymeric thiol across the maleimide double bond. This concept is demonstrated using SEC-MALLS and UV−vis spectroscopy measurements.

Introduction

During the past several years, reversible addition−fragmentation chain transfer (RAFT) polymerization has proven itself to be an exceptionally versatile controlled/"living" free radical polymerization (CRP) technique. It has been shown to be applicable to the controlled polymerization of a wide range of monomers, under many different conditions to yield materials with predetermined molecular weights, narrow molecular weight distributions (MWD), and advanced architectures.[1−6] One particularly important feature of RAFT is that it allows synthesis of polymers with terminal or internal thiocarbonylthio functionality that can ultimately be reduced, producing an ω-terminal thiol. This feature of RAFT makes it a useful synthetic tool allowing for potential tailored design and preparation of novel polymeric bioconjugates, polymer−drug conjugates, and polymers with fluorescent labels.

Our research group has a long-standing interest in the preparation of water-soluble polymers via aqueous RAFT polymerization.[3] To date, the controlled RAFT polymerization of anionic,[7−9] cationic,[10,11] zwitterionic,[12,13] and neutral[12−32] acrylamido monomers has been reported by several laboratories in both organic and aqueous media employing a variety of chain transfer agents (CTA) including xanthates,[30] dithiocarbamates,[14,23] trithiocarbonates,[15,22,32] and dithioesters.[1,7−13,16−21,24−29,31] All of these CTAs can afford control over the molecular weight and yield (co)polymers with low polydispersity (M_w/M_n) values under appropriate conditions. We recently developed conditions allowing for the controlled room-temperature RAFT polymerization of *N*-isopropylacrylamide (NIPAM) in *N*,*N*-dimethylformamide (DMF) employing a conventional azo-initiator, namely, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) as the primary source of radicals and the trithiocarbonate-based CTA, 2-dodecylsulfanylthiocarbonylsulfanyl-2-methyl propionic acid (DMP) (Scheme 1).[15] These polymerizations remained controlled even at monomer conversions exceeding 90% and bore all the characteristics of a controlled/"living" system.

Although the RAFT polymerizations of several monomer classes have been exhaustively reported, the synthetic utility of the well-defined telechelic or α,ω-functionality inherent to polymers prepared by RAFT polymerization, though mentioned in seminal RAFT studies,[33] has yet to be extensively exploited. Okano et al. employed 2-ethanolamine in the aminolysis of dithioester-terminal poly(*N*-isopropylacrylamide-*co*-*N*,*N*-dimethylacrylamide) (PID) and later reacted either iodoethanol or *N*-(1-pyrene)maleimide (PM) with the previously formed polymeric thiols.[34] While this method allows for the functionalization of the polymeric chain ends, it requires oxygen-free conditions, and the formation of disulfide polymer species is possible. Our laboratories reported the first preparation of transition metal nanoparticles and brushes via in situ reduction of telechelic dithioesters with $NaBH_4$ on a variety of RAFT-produced polymers.[35,36] Later, the same technique was utilized to prepare thermoresponsive nanoparticles.[37] Perrier et al. recently reported the ability to remove the thiocarbonylthio moiety from a poly(methyl methacrylate) (PMMA) macroCTA by flooding the system with an excess of radicals, thus regenerating the small-molecule RAFT agent and "capping" the polymer chain end with a functionalized radical fragment.[38] Rimmer et al. reported the preparation of highly branched poly(NIPAM) with terminal imidazole moieties via the controlled copolymerization of NIPAM with a novel dithioester-based CTA containing an imidazole Z-group and a 4-methylstyrene R-group.[39,40]

When the thiocarbonylthio moiety is reduced to a thiol, it becomes readily available for reaction with a variety of

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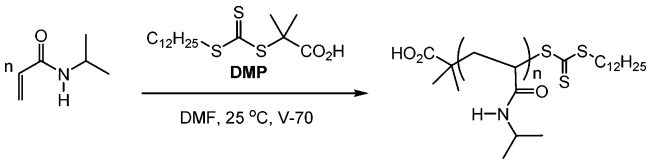
Scheme 1. Synthetic Pathway for the Room-Temperature RAFT Polymerization of *N*-Isopropylacrylamide



functionalities, including other thiols (to form disulfides), maleimides, and iodo-substituted alkyl moieties. These types of thiol-based coupling reactions are well-established and have been used for several years in fields such as biochemistry and molecular biology to easily label proteins and other thiol-containing species that are common in biological systems. This chemistry has also been employed in the synthesis of polymeric bioconjugates. For example, Tsuchida et al. recently reported the facile conjugation of poly(ethylene glycol) (PEG) to an albumin-based synthetic hemoprotein, HSA-FeXP, that functions as an artificial oxygen transporter.[41] The authors found that in vivo circulation of HSA-FeXP increased through malemide/thiol coupling of PEG to HSA-FeXP. Thibaudeau and co-workers reported the preparation of a series of novel maleimido-functionalized insulin conjugates and their facile conjugation to Cys34 of human serum albumin (HSA). These conjugates showed promising insulin release profiles in subsequent in vivo studies with diabetic rats.[42] The use of thiol−maleimide coupling reactions has also been heavily employed in the preparation of immuno−liposome conjugates (i.e., protein−lipid conjugates).[43−46] More recently, Fleiner et al. employed a similar method and reported the effective conjugation of thiol-functionalized immunoglobulin G (IgG) antibodies and bovine serum albumin (BSA) to maleimido-functionalized lipids to form immuno-labeled liposome conjugates.[47] The efficacy of thiol-based chain-end coupling reactions directly lends itself to applicability of RAFT-generated polymers in pharmaceutics and drug delivery and allows for easy conjugation under a variety of potential reaction conditions, especially aqueous-based systems. Noting the well-defined chain-end functionality that the RAFT process affords, the synthetic utility of thiocarbonylthio chain ends for potential postpolymerization modification/conjugation with reactive dyes has come to our attention. Building on this key feature inherent to RAFT, we report herein the conjugation of a fluorescent maleimide species, namely *N*-(1-pyrenyl)maleimide, to a RAFT-generated, thiol-terminated poly(*N*-isopropylacrylamide) (PNIPAM) homopolymer in the presence of a tris-(2-carboxyethyl)phosphine·HCl (TCEP).

Experimental Section

Materials. All reagents were purchased from Aldrich and used without further purification unless noted otherwise.

RAFT Polymerization of NIPAM. Nearly monodisperse PNIPAM (M_n = 39 500 g/mol, M_w/M_n = 1.07) was prepared in *N*,*N*′-dimethylformamide (DMF), employing 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) as the primary source of radicals and the trithiocarbonate-based CTA, 2-dodecylsulfanylthiocarbonylsulfanyl-2-methyl propionic acid (DMP) (Scheme 1) according to the previously published procedure.[15] The polymerization was conducted at 25 °C with a $[CTA]_o/[I]_o$ ratio of 5/1 under a nitrogen atmosphere at 33 wt % monomer in a septum-sealed flask. The CTA/monomer ratio ($[CTA]_o/[M]_o$ = 1/465) was such that the theoretical M_n at 100% conversion was 52 500 g/mol.

Instrumentation. PNIPAM and its conjugates with PM were characterized directly by SEC (DMF eluent, 0.5 mL/min, 60 °C, Polymer Labs PL gel 5-μm mixed C column, Viscotek-TDA (302 RI, viscosity, and 7 mW 90° and 7° true low-angle and light scattering detectors, λ = 670 nm)). The d*n*/d*c* of PNIPAM was determined to be 0.0731 mL/g at 632.8 nm in DMF at 60 °C using a Viscotek refractometer and Omnisec software. The presence of pyrene-labeled end groups was confirmed by comparing the area of the UV signal (λ = 340 nm) of the unmodified polymeric thiol (PNIPAM-SH) to the pyrene-labeled polymer (PNIPAM-PM) while maintaining normalized RI signals for both polymers.

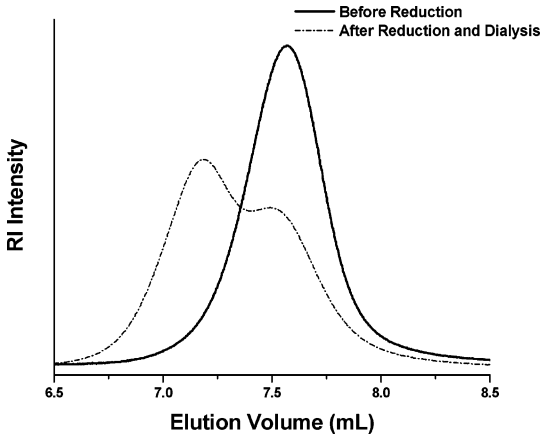


Figure 1. PNIPAM-TTC before reduction with $NaBH_4$ and the mixture of PNIPAM-SH with PNIPAM-S-S-PNIPAM after reduction and dialysis.

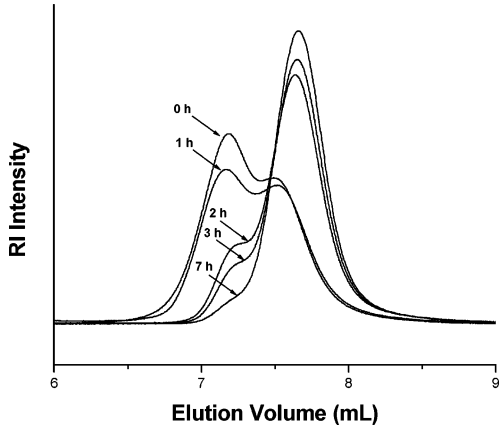


Figure 2. SEC traces showing the reduction of coupled PNIPAM-S-S-PNIPAM with TCEP at different time intervals.

Conjugation of Pyrene Maleimide to PNIPAM. To a 50-mL round-bottom flask were added PNIPAM homopolymer (PNIPAM-TTC) (M_n = 39 500 g/mol, M_w/M_n = 1.07) and 15 mL of deionized water. The resulting solution was further diluted with an additional 15 mL solution of 1 M $NaBH_4$, and the mixture was allowed to react for 2 h. Following reduction, the homopolymer solution was dialyzed against water for 3 days and subsequently lyophilized. The resulting dried polymer was then dissolved in DMF, and a solution of tris(2-carboxyethyl)phosphine (TCEP) in DMF was added to yield a 150:1 mole ratio of TCEP to polymer. This solution was allowed to react for 24 h, after which time it was charged with a solution of *N*-(1-pyrenyl)-maleimide (PM) in DMF to yield a 150:1 mole ratio of PM to polymeric thiol (PNIPAM-SH). A catalytic quantity of ethylenediamine was added, and the reaction mixture was allowed to react for 24 h at 50 °C. PNIPAM-PM was isolated from the reaction mixture by adding water to the solution to precipitate residual unconjugated PM. The resulting heterogeneous mixture was filtered with a 0.22-μm membrane filter to remove precipitated PM and produce a clear polymer solution. The resulting solution was dialyzed for 3 days in water using 6000−8000 MWCO dialysis tubing. The purified polymer was then isolated by lyophilization and characterized by SEC using online UV and RI detectors.

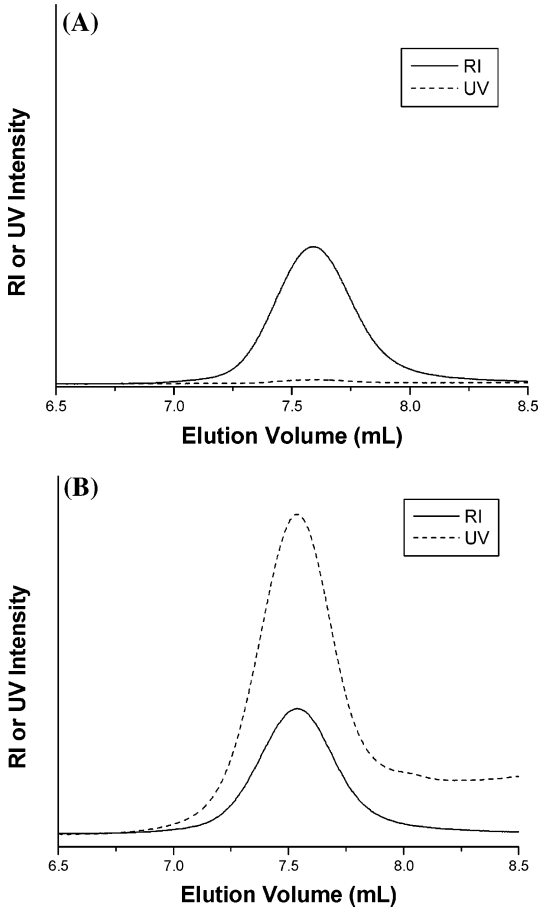


Figure 3. (A) RI and UV SEC traces of unmodified PNIPAM-SH before addition of *N*-(1-pyrenyl)maleimide. (B) RI and UV SEC traces of pyrene-labeled PNIPAM (PNIPAM-PM) after addition of *N*-(1-pyrenyl)maleimide.

Determination of Degree of Conjugation. Lyophilized poly(NIPAM)-S-PM (30.0 mg, 0.775 μmol) was added to a scintillation vial and subsequently dissolved in a known amount of DMF (1 mL) to form a 775.0 μM solution. The resulting polymer solution was characterized by SEC in DMF, and the respective RI and UV (λ = 340 nm) peak areas for the conjugated polymer were obtained (Figure 3B). The exact concentration of PNIPAM (774.0 μM) was easily obtained from the RI signal, using the previously reported specific refractive index increment for PNIPAM in DMF of 0.0731 mL/g.[14] To determine the concentration of PM associated with PNIPAM, a PM calibration curve was generated by subjecting multiple known concentrations of PM to SEC and plotting their corresponding UV peak areas at 340 nm. A linear response was observed between the PM concentration and the resulting UV peak area (Figure 4). The concentration of PM associated with PNIPAM (641 μM) was subsequently obtained by comparison of the polymer conjugate UV peak area (λ = 340 nm) to the PM calibration curve obtained at 340 nm.

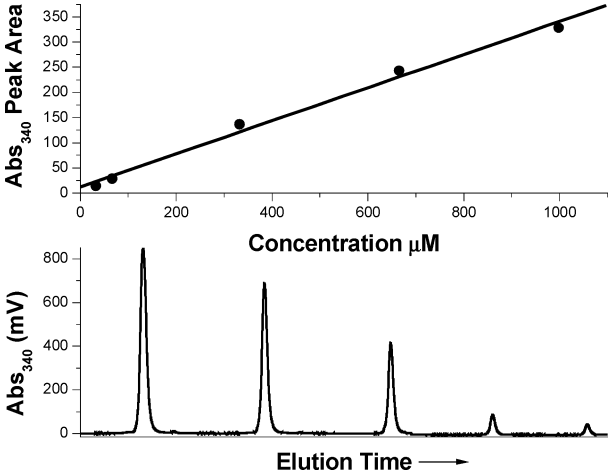
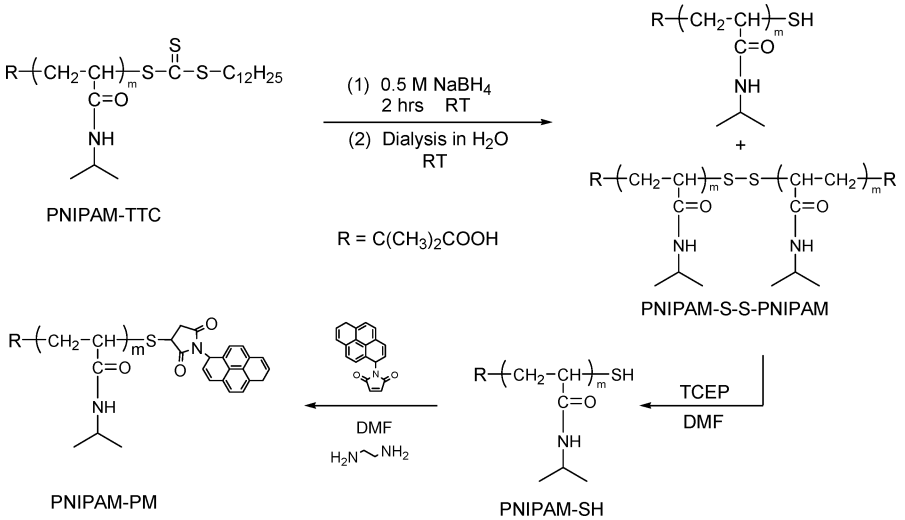


Figure 4. PM calibration curve (top) obtained by SEC−UV chromatograms of PM at multiple concentrations (bottom).

Results and Discussion

The synthesis of pyrene-labeled PNIPAM is outlined in Scheme 2. Upon reduction of the trithiocarbonate functionality with aqueous $NaBH_4$ and purification via dialysis, coupling between polymeric thiols is observed. Figure 1 shows GPC traces for both the unmodified PNIPAM (PNIPAM-TTC) and PNIPAM-TTC following reduction with $NaBH_4$ and isolation via dialysis. From the overlaid chromatograms, it is evident that, following the initial reduction with $NaBH_4$ and subsequent purification via dialysis, polymeric disulfides, namely, PNIPAM-S-S-PNIPAM, are formed. However, coupling can be easily reversed by the addition of a weak reducing agent. Figure 2 shows the molecular weight distributions for the coupled PNIPAM at several different time intervals following the addition of TCEP. The progress of this reductive cleavage reaction is evidenced by the shift of a bimodal MWD to a unimodal MWD at the elution volume of the original RAFT-

Scheme 2. Synthetic Pathway for the Reduction of Trithiocarbonate End-Groups and Conjugation with *N*-(1-pyrenyl)Maleimide



generated polymer (Figure 2). Figure 3A shows RI and UV traces for the unimodal distribution of polymeric thiols obtained in the presence of TCEP before the flask was charged with a solution of PM. Strong refractive index signals are observed for both polymers; however, prior to modification, PNIPAM shows negligible UV absorbance at 340 nm (Figure 3A). Upon reaction with PM, an intense UV signal associated with polymer elution is observed (Figure 3B), suggesting that the PNIPAM-SH chains were successfully modified with the pyrene label. The degree of PM conjugation to PNIPAM-SH was obtained using a calibration curve composed of the UV peak areas ($\lambda = 340$ nm) at specific PM concentrations and was determined to be 83.0%.

Conclusions

The synthesis of PNIPAM with controlled molecular weight and narrow MWD was accomplished using the trithiocarbonate DMP as the RAFT agent at room temperature in DMF. The resultant polymer retained a trithiocarbonate functionality at the chain end which, upon reduction and purification, resulted in a mixture of unimeric and dimeric polymer species. The dimeric disulfide species were easily reduced with TCEP and subsequently conjugated with PM to yield fluorescently labeled polymers. The degree of conjugation of PM to PNIPAM-SH was measured by online SEC-RI-UV detection and was shown to be approximately 83.0%. The exploitation of the thiocarbonylthio moiety to form polymeric thiols and the subsequent attachment of fluorescent labels represents a significant step toward the facile synthesis and characterization of well-defined fluorescently labeled polymers and demonstrates the potential use of RAFT in biotechnology and drug delivery applications.

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